The PNPLA3 I148M variant promotes lipid-induced hepatocyte secretion of CXC chemokines establishing a tumorigenic milieu

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Abstract: The I148M variant of the Patatin-like phospholipase domain-containing 3 (PNPLA3) protein is associated with an increased risk for liver inflammation and hepatocellular carcinoma (HCC), but the underlying mechanism is unknown. We hypothesized that enhanced CXC chemokine secretion mediates hepatic inflammation that accelerates development of HCC. Expandable primary human (upcyte®) hepatocytes and human PLC/PRF/5 hepatoma cells were lentivirally transduced with both PNPLA3 I148M variants and stimulated with lipids. Cytokine levels in culture supernatant and patient sera (n = 80) were analyzed by ELISA. Supernatants were assessed in transmigration experiments, tube formation, and proliferation assays. In vitro, lipid stimulation of transduced hepatocytes dose-dependently induced the production of interleukin-8 and CXCL1 in hepatocytes carrying the PNPLA3 148M variant. In line, sera from PNPLA3 148M-positive patients with alcoholic liver cirrhosis contained higher levels of interleukin-8 and CXCL1 than patients with wild-type PNPLA3. Supernatants from lipid-stimulated hepatocytes with the PNPLA3 148M variant induced enhanced migration of white blood cells, angiogenesis, and cell proliferation in comparison with supernatants from wild-type hepatocytes via CXC receptors 1 and 2. Increased production of interleukin-8 and CXCL1 by hepatocytes carrying the PNPLA3 148M variant contributes to a pro-inflammatory and tumorigenic milieu in patients with alcoholic liver disease. KEY MESSAGES: The PNPLA3 148M variant is associated with cirrhosis and hepatocellular carcinoma. Lipid stimulation of hepatocytes with this variant induces IL-8 and CXCL1. Supernatants from hepatocytes with this variant promote migration and angiogenesis. Sera from patients with this variant contained enhanced levels of IL-8 and CXCL1. The PNPLA3 148M variant contributes to a tumorigenic milieu via IL-8 and CXCL1.

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The PNPLA3 I148M variant promotes lipid induced hepatocyte secretion of CXC chemokines establishing a tumorigenic milieu

Hans Dieter Nischalke[1*], Philipp Lutz[1*], Eva Bartok[2], Benjamin Krämer[1], Bettina Langhans[1], Regina Frizler[1], Thomas Berg[3], Jochen Hampe[4], Stephan Buch[4], Christian Datz[5], Felix Stickel[6], Gunther Hartmann[2], Christian P. Strassburg[1], Jacob Nattermann[1§] and Ulrich Spengler[1§]

[1]Department of Internal Medicine I, University Hospital, University of Bonn, Germany

[2]Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital, University of Bonn, Germany

[3]Department of Gastroenterology, University Hospital Leipzig, Germany

[4]Medical Department 1, University Hospital Dresden, TU Dresden, Germany

[5]Department of Internal Medicine, Hospital Oberndorf, Teaching Hospital of the Paracelsus Private University of Salzburg, Austria

[6]Department of Gastroenterology and Hepatology, University Hospital of Zürich, Switzerland

[*] HDN and PL contributed equally to the study and the manuscript

[§] JN and US contributed equally to the study and the manuscript and assume equal responsibility for senior authorship

The authors declare that they have no competing interests.

Corresponding author:

Dr. Hans Dieter Nischalke; Department of Internal Medicine I, University of Bonn; Sigmund-Freud-Strasse 25; D – 53127 Bonn, Germany;
Email: nischalke@ukbonn.de; Phone: +49 228 287 51416;

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Abstract

Background: The I148M variant of the Patatin-like phospholipase domain-containing 3 (PNPLA3) protein is associated with an increased risk for liver inflammation and hepatocellular carcinoma (HCC), but the underlying mechanism is unknown. We hypothesized that enhanced CXC chemokine secretion mediates hepatic inflammation that accelerates development of HCC.

Methods: Expandable primary human (Upcyte®) hepatocytes and human PLC/PRF/5 hepatoma cells were lentivirally transduced with both PNPLA3 I148M variants and stimulated with lipids. Cytokine levels in culture supernatant and patients sera (n=80) were analysed by ELISA. Supernatants were assessed in transmigration experiments, tube formation and proliferation assays.

Results: *In-vitro*, lipid stimulation of transduced hepatocytes dose-dependently induced the production of interleukin-8 and CXCL1 in hepatocytes carrying the PNPLA3 148M variant. In line, sera from PNPLA3 148M positive patients with alcoholic liver cirrhosis contained higher levels of interleukin-8 and CXCL1 than patients with wild-type PNPLA3. Supernatants from lipid-stimulated hepatocytes with the PNPLA3 148M variant induced enhanced migration of white blood cells, angiogenesis and cell proliferation in comparison to supernatants from wild-type hepatocytes via CXC receptor 1 and 2.

Conclusion: Increased production of interleukin-8 and CXCL1 by hepatocytes carrying the PNPLA3 148M variant contributes to a pro-inflammatory and tumorigenic milieu in patients with alcoholic liver disease.

Keywords: PNPLA3 148M, IL-8, CXCL1, cirrhosis, HCC, alcoholic liver disease, rs738409

Introduction

Chronic liver disease is a major health burden leading to more than 1 million deaths per year in adults worldwide[1]. While the prevalence of liver diseases caused by infection with hepatitis B or C virus is likely to decline due to the availability of effective antiviral treatment, liver disease caused by alcoholic and non-alcoholic steatohepatitis is expected to become more frequent[2]. A common polymorphism in the *Patatin like phospholipase domain containing 3* (*PNPLA3*) gene, leading to an exchange from isoleucine to methionine at position 148 (rs738409), is linked to progression of both alcoholic- and non-alcoholic liver disease[3, 4]. Presence of this variant increases the risk for fibrosis/cirrhosis[5, 6] and hepatocellular carcinoma (HCC) [7–9]. However, the underlying molecular mechanisms are not yet understood.

The PNPLA3 protein acts as a triglyceride hydrolase, whose function is impaired by the I148M variant, resulting in hepatic triglycerides accumulation[10]. The presence of the 148M variant impairs the mobilization of triglycerides from lipid droplets by interfering with ubiquitylation and proteasomal degradation of the PNPLA3 protein, **which causes the disease phenotype**[11, 12]. In addition, PNPLA3 displays retinyl-palmitate lipase activity in human stellate cells (HSC)[13]. Fat accumulation in the liver is associated with increased hepatic inflammation in patients with non-alcoholic fatty liver disease[14]. Importantly, patients carrying the PNPLA3 148M variant display higher transaminase levels even after adjustment for metabolic parameters[15], suggesting that increased intrahepatic inflammatory activity contributes to enhanced risk for cirrhosis and HCC in patients with the PNPLA3 148M variant. Experimental analysis of the so far unknown mechanism is hampered, because PNPLA3 knock-out mice do not develop liver disease[16]. **Still, hepatic**

over-expression of the PNPLA3 148M variant in mice or PNPAL3 148M knock-in mice develop liver steatosis when fed high-sucrose diet[17, 18].

Histologically, the presence of the *PNPLA3* variant leads to more pronounced infiltration of leukocytes into the liver[6, 19]. Chronic inflammation precedes development of HCC in most cases[20]. Hepatocytes respond to lipids with production of significant amounts of inflammatory cytokines[21].

Therefore, we tested if the presence of the *PNPLA3* 148M variant induces release of an inflammatory cytokine pattern in hepatocytes upon exposure to lipids, which may create a tumorigenic milieu in the liver. Thus, we analyzed expandable primary human hepatocytes and PLC/PRF/5 hepatoma cells lentivirally transduced with both *PNPLA3* variants.

Materials and Methods

Cell culture

Upcyte® human hepatocytes (UHH), a source of expandable, genetically-modified, primary, non-malignant hepatocytes, (upcyte technologies GmbH, Hamburg, Germany) are lentivirally transduced so that a switch between controlled proliferation and the differentiated primary phenotype can be induced by specific media without inducing immortalization. For proliferation, UHH were cultivated in collagen I-coated T-75 flasks with Hepatocyte Culture Medium (HCM) containing Supplement A, Supplement B and 2 mM L-glutamine (upcyte® technologies GmbH) as recommended by the manufacturer. After reaching confluency, cells were detached with trypsin and reseeded. To stop proliferation and induce re-differentiation to their primary phenotype, UHH were plated at a density of 6000 cells/well in a collagen I-

coated 48-well plate and cultured in High Performance Medium (HPM=HCM without Supplement B) for 3 days before and during experiments. **UHH cells from passage 7-10 were used.** In addition, PLC/PRF/5 hepatoma cells (LGC Standards GmbH, Wesel, Germany) were cultured in RPMI 1640 medium containing L-glutamine (Thermo Fisher, Schwerte, Germany), penicillin/streptomycin (PAN-Biotech GmbH, Aidenbach, Germany) and 10% fetal calf serum (FCS, Biochrom, Berlin, Germany) as a further control and cell model, which does not carry the limitations in cell proliferation of primary or expandable primary hepatocytes. HepG2 cells (LGC Standards GmbH) were cultured in RPMI 1640 medium containing L-glutamine, penicillin/streptomycin and 10% fetal calf serum.

Molecular cloning, production and transduction of lentiviral constructs

A lentiviral vector pLenti-GIII-CMV-GFP-2A-Puro containing cDNA coding for the PNPLA3 148M variant (Applied Biological Materials Inc., Vancouver, Canada) was converted to the 148I (G444A) consensus sequence using Quikchange Mutagenesis (Primer 5'-tggtatgttcctgcttcatacctttctacagtggc-3', 5'-gccactgtagaaaggtatgaagcaggaacatacca-3'). Lentivirus was then generated via co-transfection of the respective lentiviral plasmid with the second-generation packaging constructs pCMV delta R8.2 and pMD2.G /VSV-G (Trono Lab, Addgene, Cambridge, MA, USA) in HEK293FT cells (ThermoFisher Scientific, Carlsbad, California, USA) as previously described[22]. Transduction of upcyte® human hepatocytes and PLC/PRF/5 hepatoma cells was performed at MOI 10. Cells were selected with 2µg/mL puromycin 96h after transduction. PNPLA3 overexpression was validated on the mRNA level by quantitative RT-PCR and on the protein level by western blot. Both showed a close correlation to the expression of the GFP reporter (supplementary figure 1). To ensure comparable PNPLA3 148I and PNPLA3 148M

expression levels, we controlled GFP-expression by flow cytometry at each passage of cells. If the GFP-expression differed by more than 10%, it was adjusted by changing the puromycin concentration of the selection medium.

Cytokine array

To gain insights in differential cytokine regulation by upcyte® human hepatocytes (UHH) lentivirally transduced with PNPLA3 wildtype or I148M variant, we analysed supernatants with the Human XL Cytokine Array Kit (Bio-Techne, Wiesbaden, Germany) according to the manufacturer's instructions.

Cell stimulation

Human hepatocytes were stimulated overnight with different concentrations of fetal calf serum (FCS) (1-20%), human serum albumin (HSA) (4%) (Octapharma, Langenfeld Germany) and palmitate (50µM). Lipid accumulation increased in cells stimulated with FCS when the PNPLA3 148M variant was present (supplementary figure 2). To test for possible involvement of endoplasmatic reticulum (ER) stress, 5µM 4µ8C, an inhibitor of inositol requiring enzyme 1 (IRE1), and 300nM GSK2606414, an inhibitor of Protein Kinase RNA-like Endoplasmic Reticulum Kinase (PERK), respectively (both Merck Chemicals GmbH, Darmstadt, Germany), were added to some experiments 30 minutes before stimulation. As a positive control, ER stress was induced by incubating the cells with 2µg/ml tunicamycin.

Determination of CXC chemokine levels

CXCL8/IL-8, CXCL1, CXCL4 and CXCL5 concentrations in patient sera and/or cell culture supernatants were assessed by the human IL-8 ELISA MAX™ (BioLegend, Fell, Germany), the human CXCL1/GROalpha, CXCL4/PF4 and CXCL5 DuoSet ELISA, (all Bio-Techne) according to the manufacturer's protocols.

Leukocyte transmigration assay

A 96-well (3.0µm pore size) transmigration chamber (Corning, Wiesbaden, Germany) was used, adding 235µL of 1:5 diluted supernatants from FCS (15%) stimulated PLC/PRF/5 cells carrying either PNPLA3 148I or M variant to each well. RPMI medium alone was used as negative control. Then, $2x10^5$ white blood cells (WBC), which were obtained from healthy donors after red blood cell lysis[23], were added in 75µl RPMI medium to the top chamber. CXCR1 and CXCR2 blocking antibodies (clone 42705 and 48311, Bio-Techne) or an appropriate isotype control (clone 20102, Bio-Techne) were added at 10µl/ml to the WBC 30 min prior to incubation. Plates were incubated at 37°C for 2.5h, and cells migrating to the bottom chamber were quantified and classified into granulocytes, monocytes and lymphocytes based on size and granularity by flow cytometry on a BD FACS Canto II flow cytometer.

Tube Formation Assay

Measurements of capillary-like tube formation of HUVEC (PromoCell, Heidelberg) were performed with a Matrigel™ *in vitro* assay to assess angiogenetic potential of cell culture supernatants. HUVEC were cultured in starvation medium (Endothelial Cell Growth Medium without supplements; Provitro, Berlin) containing 1% FCS overnight prior to the experiment. Growth factor-reduced Matrigel™ (BD Biosciences, Heidelberg) was plated onto 18-well Angiogenesis µ-Slides (10µL/well; IBIDI,

Munich) and incubated at 37°C for 1h. HUVEC were seeded in 40µl starvation medium containing 1% FCS at 6000/well. After 2h, medium was replaced by the respective supernatants from stimulation experiments with or without blocking antibodies against CXCR1 and CXCR2 at a final concentration of 10µg/ml. We used supernatants from UHH, because HUVEC cells as primary cells require additional factors for growth. Images were captured using a Axiovert 200M inverted microscope. Tube formation was quantified by counting the number of completely closed ring structures.

Proliferation assay

Proliferation assays were performed with the bromodeoxyuridine (BRDU) Flow Kit (BD Biosciences) according to the manufacturer's instructions. In brief, HepG2 cells ($2x10^4$/well) were seeded and incubated for 24h in starvation medium (RPMI 1640 +1% FCS) in a 48-well plate at 37°C and 5% CO_2. The starvation medium was then replaced by conditioned supernatants. Incubation was continued overnight. CXCR1 and CXCR2 were blocked by antibodies at 10µl/ml 30 min prior to incubation, using an appropriate isotype as control. Finally, 10µM BRDU were added for 30 min. Cells were then harvested, fixed, stained and analysed on a BD Canto II Flow Cytometer.

Patients

We collected serum from patients with alcoholic liver cirrhosis (n=80) and healthy controls (n=53) (table 1). Determination of the *PNPLA3* rs738409 polymorphism was performed as previously described[7]

Statistical analyses

Experiments were performed in triplicates and repeated at least **three times.**

Quantitative data were analyzed using the non-parametric Mann-Whitney-U

test when comparing two and the non-parametric ANOVA (Friedman test) when comparing several groups. Test multiplicity was controlled by a false discovery rate (FDR) procedure (Benjamini, Krieger, Yetukieli) using GraphPad Prism Version 7 (GraphPad Software Inc, San Diego, CA, USA). FDR-adjusted P values <0.05 were considered statistically significant

Results

Hepatocytes carrying the PNPLA3 148M variant produce higher levels of CXCL1 and IL-8

To analyze the functional role of the PNPLA3 I148M polymorphism at the molecular level, we lentivirally transduced both the wildtype (148I) and the 148M variant into expandable primary human hepatocytes (upcyte® hepatocytes, UHH). After re-differentiation of the cells to their primary phenotype, we performed a cytokine screen which indicated enhanced secretion of CXCL1, CXCL4, CXCL5, CXCL8/IL-8, while CXCL10 was apparently downregulated. Subsequent quantitative studies by ELISA confirmed significantly increased production of CXCL1 and IL-8 in UHH with the PNPLA3 148M variant (Figure 1 A,B). By contrast, differences in the cytokine screen between hepatocytes carrying the 148I or 148M PNPLA3 variant were not reproduced concerning CXCL4 (810pg/ml vs 901pg/ml; p=0.22,), CXCL5 (39.8ng/ml vs 39.0ng/ml; p=0.96) or CXCL10 (497pg/ml vs 474pg/ml; p=0.65). To check whether increased IL-8 and CXCL1 production could be confirmed in a standard hepatoma line, we transduced the PNPLA3 148M variant into PLC/PRF/5 human hepatoma cells, which unlike many other hepatoma lines such as Huh7 or HepG2 do not constitutively carry the PNPLA3 148M variant. Comparing the genetic PNPLA3

variants in transduced PLC/PRF/5 human hepatoma cells, the same pattern of differentially produced IL-8 and CXCL1 was observed (Figure 1 C,D). **Conversely, when we silenced PNPLA3 in Huh7 liver cells which constitutively carry the PNPLA3 148M/M variant, we found decreased secretion of IL-8 (supplementary figure 3).**

To analyze if IL-8 and CXCL1 production was stimulated by exposure to lipids, we added palmitate as well as lipid carriers (FCS and HSA) to the transduced hepatocytes. Chemokine levels in PNPLA3 148M positive cells significantly exceeded the amounts in cells transduced with PNPLA3 wildtype (148I) (Figure 2 A-D). Exposure to lipids induced release of IL-8 and CXCL1 in a dose dependent fashion (Figure 2 E, F).

Patients carrying a PNPLA3 148M allele display higher circulating levels of CXC-chemokines IL-8 and CXCL1, but not CXCL5

To verify if the PNPLA3 148M variant also leads to differences in chemokine levels *in-vivo*, we measured serum levels of IL-8, CXCL1 and CXCL5 both in healthy controls and patients with alcoholic liver cirrhosis stratified for PNPLA3 genotype (demographic and clinical details are given in table 1). As displayed in figure 3**,** we found comparatively low serum levels independent of the PNPLA3 genotype in healthy controls, while patients with liver cirrhosis revealed significantly increased IL-8 (p=0.011) and CXCL1 (p=0.012) serum levels in carriers of the PNPLA3 148M variant. These results remained significant after correction for severity of liver cirrhosis (Child-Pugh stage) or presence of HCC (n=14) (IL-8 p=0.03, CXCL1 p=0.03). By contrast, CXCL5 levels did not differ between carriers of the PNPLA3 148I and 148M variants.

Differences in IL-8 and CXCL1 secretion cannot be attributed to increased ER stress in hepatocytes with the PNPLA3 148M variant

Lipid overload in hepatic cells induces endoplasmatic reticulum (ER) stress[24], which might be aggravated by the accumulation of the PNPLA3 protein due to its impaired degradation in presence of the PNPLA3 148M variant[11]. We suspected that ER stress might cause the increased IL-8 and CXCL1 secretion in hepatocytes carrying the PNPLA3 148M variant. Therefore, we treated 15% FCS stimulated PLC/PRF/5 with either 4µ8C, an inhibitor of IRE1, or GSK2606414, an inhibitor of PERK, which block two major downstream-signaling pathways linking ER stress to cytokine regulation. However, although the inhibitors reduced IL-8 production in response to tunicamycin, a positive control agent known to induce ER stress, none of the inhibitors blocked the excess of IL-8 production seen in hepatocytes carrying the PNPLA3 148M variant (Figure 4). Thus, differences in ER stress are unlikely to explain differential IL-8 production by hepatocytes depending on the PNPLA3 148 genotype.

Supernatants from PNPLA3 148M positive hepatocytes induced enhanced chemotaxis in leukocytes

To check if differential cytokine expression by hepatocytes carrying the two different PNPLA3 variants contributed to a pro-inflammatory and ultimately oncogenic milieu, we first investigated whether supernatants from stimulated, PNPLA3-transduced PLC/PRF/5 cells were chemotactic for leukocytes using a transmigration assay with conditioned supernatants and leukocytes from healthy donors. Both supernatants from hepatocytes with and without the PNPLA3 148M variant induced migration of leukocytes, but supernatants from hepatocytes carrying the PNPLA3 148M variant attracted significantly more **leukocytes (Figure 5A), in particular granulocytes**

(Figure 5B-D). Blocking CXCR1 and CXCR2, the shared receptors for CXCL1 and IL-8, inhibited the enhanced migration towards supernatant from cells carrying the PNPLA3 148M variant.

Supernatants from hepatocytes with a PNPLA3 I148M variant are angiogenic

To assess if the PNPLA3 I148M polymorphism promotes angiogenesis, we used supernatants from lipid-stimulated UHH in a tube formation assay. Supernatant from lipid-stimulated UHH with the 148M variant led to increased tube formation as compared to supernatants from UHH with the 148I variant or from untransduced UHH (Figure 6 A-C). Of note, this difference was abolished by blocking CXCR1 and CXCR2, suggesting that CXC-chemokines such as IL-8 and CXCL1 mediate enhanced angiogenesis in presence of the PNPLA3 I148M variant.

Supernatants from hepatocytes with a PNPLA3 I148M variant increase the proliferation of HepG2 cells

We then wondered whether supernatants of hepatocytes carrying the PNPLA3 I148M variant might increase proliferation in hepatocytes that have already undergone malignant transformation, such as HepG2 cells. Proliferation of HepG2 cells was induced to a greater extent by supernatants from FCS-stimulated PLC/PRF/5 cells carrying the PNPLA3 148M variant compared to the PNPLA3 148I wild-type. Again, this effect could be abrogated by blocking CXCR1 and CXCR2, suggesting that CXC chemokines induced by the PNPLA3 148M variant induce proliferation in susceptible hepatocytes (Figure 6D).

Discussion

Carriage of the PNPLA3 148M variant has been associated with the progression and severity of metabolic liver disease in multiple studies[25, 26]. Since patients with liver disease and the PNPLA3 148M variant are characterized by increased intrahepatic leukocyte counts[6, 19] and based on our exploratory cytokine array analysis, we hypothesized that CXC-chemokines might mediate increased liver inflammation in patients carrying the PNPLA3 148M variant by attracting leukocytes and ultimately create a tumorigenic milieu. Production of CXC-chemokines by hepatocytes in response to lipids has already been demonstrated by other groups[21]. Here, we add the information that carriage of the PNPLA3 148M variant increases IL-8 and CXCL1 production by hepatocytes in response to lipids, irrespective if the cells were exposed to free fatty acids, such as palmitate, or to lipid carriers such as FCS or HSA. **In addition to acting as a lipid carrier, albumin may induce IL-8 secretion by activating the epidermal growth factor receptor (EGFR)[27], which may explain in part the high secretion of IL-8 even in cells carrying the PNPLA3 wildtype.** As a particular strength of our study, we used genetically modified primary hepatocytes (upcyte® hepatocytes, UHH) to mimic the *in-vivo* situation as closely as possible. We found differential IL-8 and CXCL1 production both in UHH and a human hepatoma cell line (PLC/PRF/5) when they were transduced with either the PNPLA3 148I or 148M variant. **The link between production of inflammatory chemokines and carriage of the PNPLA3 148M variant was further strengthened by the fact that silencing of PNPLA3 148M in Huh7 cells reduced expression of IL-8. In line, Linden et al. described reduced production of inflammatory cytokines and decreased fibrosis when PNPLA3 was silenced in a PNPLA3 148M knock-in mouse model of hepatic steatosis[28].** Unlike previous reports from HSC, we did not detect differences in expression of CCL2 or CCL5 in relation to the PNPLA3 genotype[29]. This indicates that the PNPLA3 mutation is linked to different effects in

hepatocytes and HSC. Our *in-vitro* findings corresponded to differential IL-8/CXCL1 serum levels in patients with alcoholic liver cirrhosis when they were stratified with respect to the PNPLA3 genotype.

The mechanism linking the PNPLA3 148M variant to the increased induction of CXC chemokines remains unclear. Perturbation of the normal function of endoplasmatic reticulum (ER) leads to ER stress, which has been implicated in liver disease of different etiologies and HCC development[30] and results in activation of NFκB and production of inflammatory mediators, among others IL-8, via inositol-requiring enzyme 1 (IRE1)[31] or via PERK/CHOP signaling[32]. Lipid overload has been reported to induce chronic ER stress[24], which might be further increased by impaired degradation of the PNPLA3 protein in presence of the 148M variant[11]. We therefore hypothesized that blocking the ER stress sensors IRE1 or PERK might abrogate the enhanced IL-8 secretion in PNPLA3 148M positive hepatocytes. Contrary to our expectations, inhibition of IRE1 or PERK did not block the increased IL-8 secretion detected in PNPLA3 148M positive cells, while either inhibitor reduced IL-8 secretion induced by tunicamycin. Alternative plausible causes for enhanced IL-8 and CXCL1 induction might be oxidative stress by lipid overload or lipid toxicity[31].

Increased IL-8 and CXCL1 production in response to lipid exposure in hepatocytes with the PNPLA3 148M variant could explain several findings observed in PNPLA3 148M positive patients with fatty liver disease: increased intrahepatic leukocyte content may be due to chemotaxis by IL-8 and CXCL1, which enhance angiogenesis and proliferation, promoting malignant transformation towards development of HCC. Of note, it has been shown that HCC progresses more rapidly in patients carrying the PNPLA3 148M variant independent of other risk factors[33, 34]. Migration experiments showed that supernatants from lipid-stimulated hepatocytes induced

migration of leukocytes, in particular granulocytes, to a greater extent in carriers of the PNPLA3 148M variant than in carriers of the wild-type. Of note, this difference was dependent on the CXC chemokine receptors 1 and 2. To substantiate the connection between the PNPLA3 148M variant, CXC chemokine secretion, and promotion of hepatic malignancy, we studied supernatants from stimulated hepatocytes in angiogenesis and proliferation assays. In short, we found that supernatants from the PNPLA3 148M hepatocytes increased angiogenesis in HUVEC cells and proliferation of HepG2 cells, providing further support for the concept that this genetic variant leads to a microenvironment which promotes tumor growth. Again, increased angiogenesis and proliferation could be abolished by blocking CXCR1 and CXCR2. CXCR1 and CXCR2 are the receptors for CXCL1 and IL-8, but can also bind CXCL6 and CXCL2-3/CXCL5-6, so that we cannot completely rule out that other CXC chemokines might be involved as well[35]. For instance, CXCL5 may promote development of HCC[36, 37]. However, we did not detect a differential CXCL5 pattern neither in cell supernatants nor in patient sera.

CXC chemokines are known to be important mediators of leukocyte invasion in liver disease[38]. CXCL1 and IL-8 have already been linked to fibrosis development[39, 40]. Previously, we demonstrated that a genetic variant in the *CXCL1* gene was associated with increased serum levels of CXCL1 and that this variant was more frequent among patients with alcoholic liver cirrhosis and HCC[40]. Serum levels of both CXCL1 and CXCL8/IL-8 have been reported as biomarkers for HCC[41, 42], with serum IL-8 being also an independent predictor of survival in patients with HCC[43]. Intrahepatic expression of CXCL1 is a risk factor for mortality and recurrence of HCC[44, 45], while silencing CXCL1 reduced tumor growth in an *in-vitro* study[46]. Mechanistically, IL-8 enhances the capacity of HCC cells to form

metastases via the transcription factor forkhead box C1[47]. Unfortunately, analysis of the IL-8 dependent oncogenic effect of the PNPLA3 148M genotype in animal models is not possible, because mice lack IL-8.

A detailed knowledge of the molecular pathways involved in mediating the negative effects of the PNPLA3 mutation would help to identify targets for prevention of HCC. This is important, because lifestyle modifications offer an effective treatment by reducing body weight in patients affected by the PNPLA3 mutation[48], but the HCC risk remains elevated[49]. Because the PNPLA3 148M variant increases the risk for complications across different etiologies of liver disease[50], pharmacological modulation of such targets might be beneficial for a wide range of patients. Here, we present evidence that CXC chemokines are excessively produced in response to lipids by hepatocytes that carry the PNPLA3 148M variant, which may contribute to the development of liver cancer. Further elucidation of the mechanistic consequence of this genetic alteration will hopefully help us to progress from merely identifying patients at particular risk to targeted prophylactic interventions in order to prevent HCC.

Compliance with ethical standards

The study protocol was approved by the ethics committee of the University of Bonn (number 351/15) and complied with the ethical guidelines given by the Declaration of Helsinki. Written informed consent was given from all patients.

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Figure Legends

Fig. 1: Increased basal production of unstimulated human hepatocytes carrying the PNPLA3 148M variant.

Levels of IL-8 (A+C) and CXCL1 (B+D) in the supernatant of unstimulated upcyte® human hepatocytes (A+B) and PLC/PRF/5 (C+D) carrying the PNPLA3 148I wild-type or 148M variant after overnight incubation. **Statistical analysis with non-parametric ANOVA (Friedman test). Error bars indicate SEM.**

Fig. 2: The enhanced CXC chemokine production of human hepatocytes with the PNPLA3 148M variant increases alongside with lipid availability.

IL-8 (A, C) and CXCL1 (B, D) levels in the supernatant of upcyte® human hepatocytes cells (A, B) or PLC/PRF/5 (C, D) stratified for PNPLA3 I148M genotype after overnight stimulation with fetal calf serum (FCS), human serum albumin (HSA) or palmitate; the IL-8 (E) and CXCL1 (F) production is dose dependent of FCS concentration, as exemplarily shown for PLC/PRF/5 cells. Statistical analysis with Mann-Whitney-U test. Error bars indicate SEM.

Fig. 3: Serum levels of CXCL1 and IL-8 are increased in patients with alcoholic liver cirrhosis carrying the PNPLA3 148M variant.

Serum levels of IL-8 (A), CXCL1 (B) and CXCL5 (C) were measured by ELISA in the serum of patients with alcoholic liver cirrhosis and healthy controls stratified according to the PNPLA3 genotype. **Statistical analysis with Mann-Whitney-U test.** Error bars indicate SEM.

Fig. 4: The increased IL-8 production of hepatocytes carrying the PNPLA3 I148M variant is not mediated by PERK or IRE1

While IL-8 production after induction of ER stress in PLC/PRF/5 cells by tunicamycin as a positive control was prevented by inhibitors of PERK or IRE1, both inhibitors did not have an effect on IL-8 production induced by 15% FCS in PNPLA3 148M positive cells**. Statistical analysis with Mann-Whitney-U test.** Error bars show SEM.

Fig. 5: Leukocytes migrate preferentially versus supernatant from hepatocytes carrying the PNPLA3 148M variant.

Using a transwell chamber assay, migration of healthy human leukocytes versus 1:5 diluted supernatant from 15% FCS stimulated PLC/PRF/5 cells was assessed by flow cytometry. (A) Summary graph show the enhanced migration versus supernatant from hepatocytes carrying the PNPLA3 148M variant, which is mediated via receptors CXCR1 and CXCR2. Representative forward/sideward scatter plots of leukocytes before (B) and after migration (C+D) to the bottom well against supernatant from cells with the PNPLA3 148I (C) and 148M (D) variant. **Statistical analysis with non-parametric ANOVA (Friedman test). Error bars indicate SEM.**

Fig. 6: Supernatants from human hepatocytes carrying the PNLPA3 148M variant increase angiogenesis and proliferation.

Angiogenetic potential was assessed by a tube formation assay (A-C). Human umbilical vein endothelial cells (HUVEC) formed more tubes if treated with supernatant from stimulated upcyte® human hepatocytes carrying the PNPLA3 148M

variant compared to the wild-type in a CXCR1 and CXCR2 dependent manner. Representative microscopic images from wild-type supernatants (A) and PNPLA3 148M variant supernatants (B) and summary graph (C).

Proliferation of HepG2 cells as assessed by bromodeoxyuridine (BRDU) incorporation increased in the presence of supernatant from stimulated PLC/PRF/5 carrying the PNPLA3 148M variant compared to the 148I wild-type in a CXCR1 and CXCR2 dependent manner. (D) **Statistical analysis with non-parametric ANOVA (Friedman test). Error bars indicate SEM.**

Figure 1

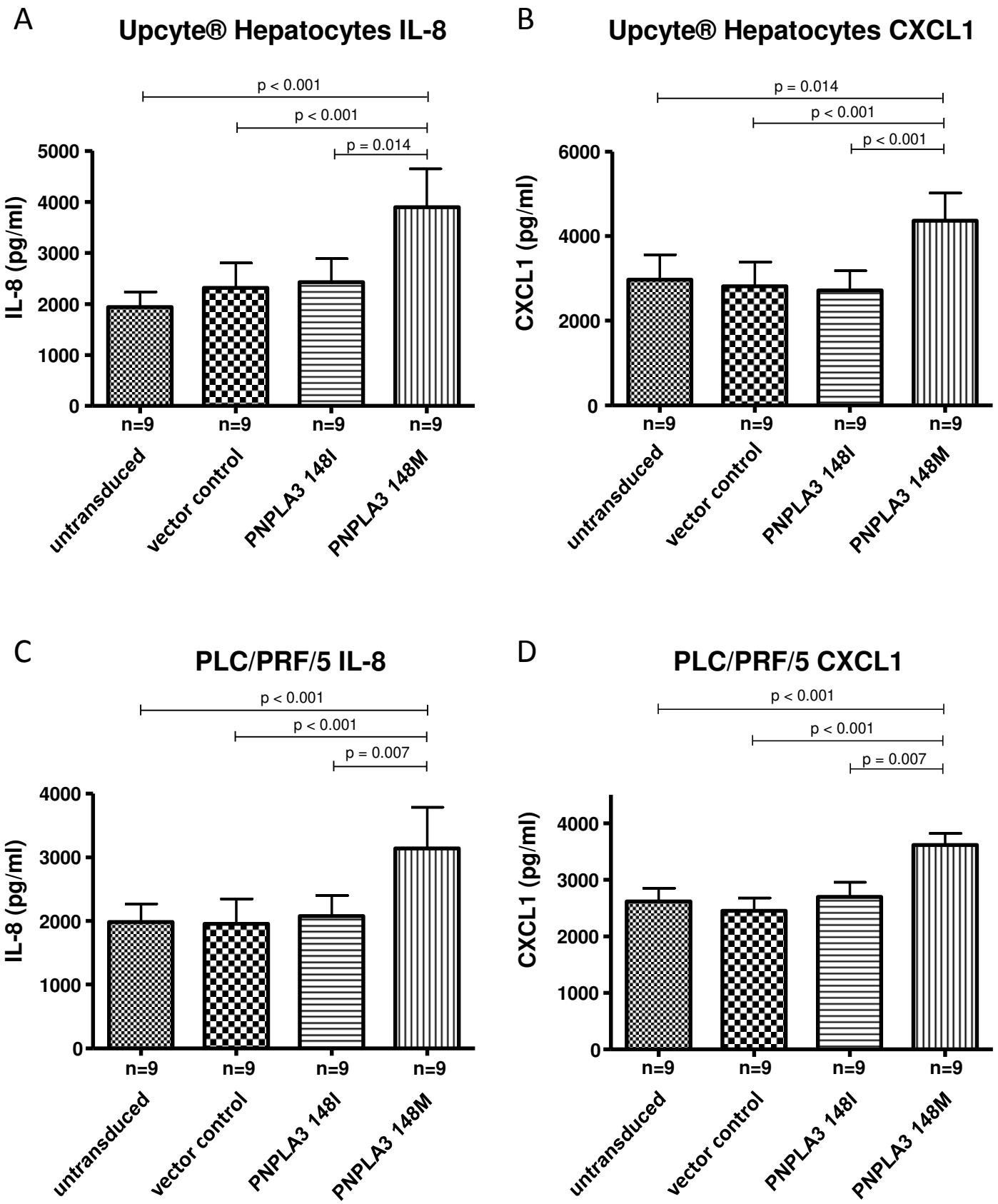
A
Upcyte® Hepatocytes IL-8
p < 0.001
p < 0.001
p = 0.014
IL-8 (pg/ml)
5000
4000
3000
2000
1000
0
n=9
n=9
n=9
n=9
untransduced
vector control
PNPLA3 148I
PNPLA3 148M
B
Upcyte® Hepatocytes CXCL1
p = 0.014
p < 0.001
p < 0.001
CXCL1 (pg/ml)
6000
4000
2000
0
n=9
n=9
n=9
n=9
untransduced
vector control
PNPLA3 148I
PNPLA3 148M
C
PLC/PRF/5 IL-8
p < 0.001
p < 0.001
p = 0.007
IL-8 (pg/ml)
4000
3000
2000
1000
0
n=9
n=9
n=9
n=9
untransduced
vector control
PNPLA3 148I
PNPLA3 148M
D
PLC/PRF/5 CXCL1
p < 0.001
p < 0.001
p = 0.007
CXCL1 (pg/ml)
4000
3000
2000
1000
0
n=9
n=9
n=9
n=9
untransduced
vector control
PNPLA3 148I
PNPLA3 148M

Figure 2

Figure 2

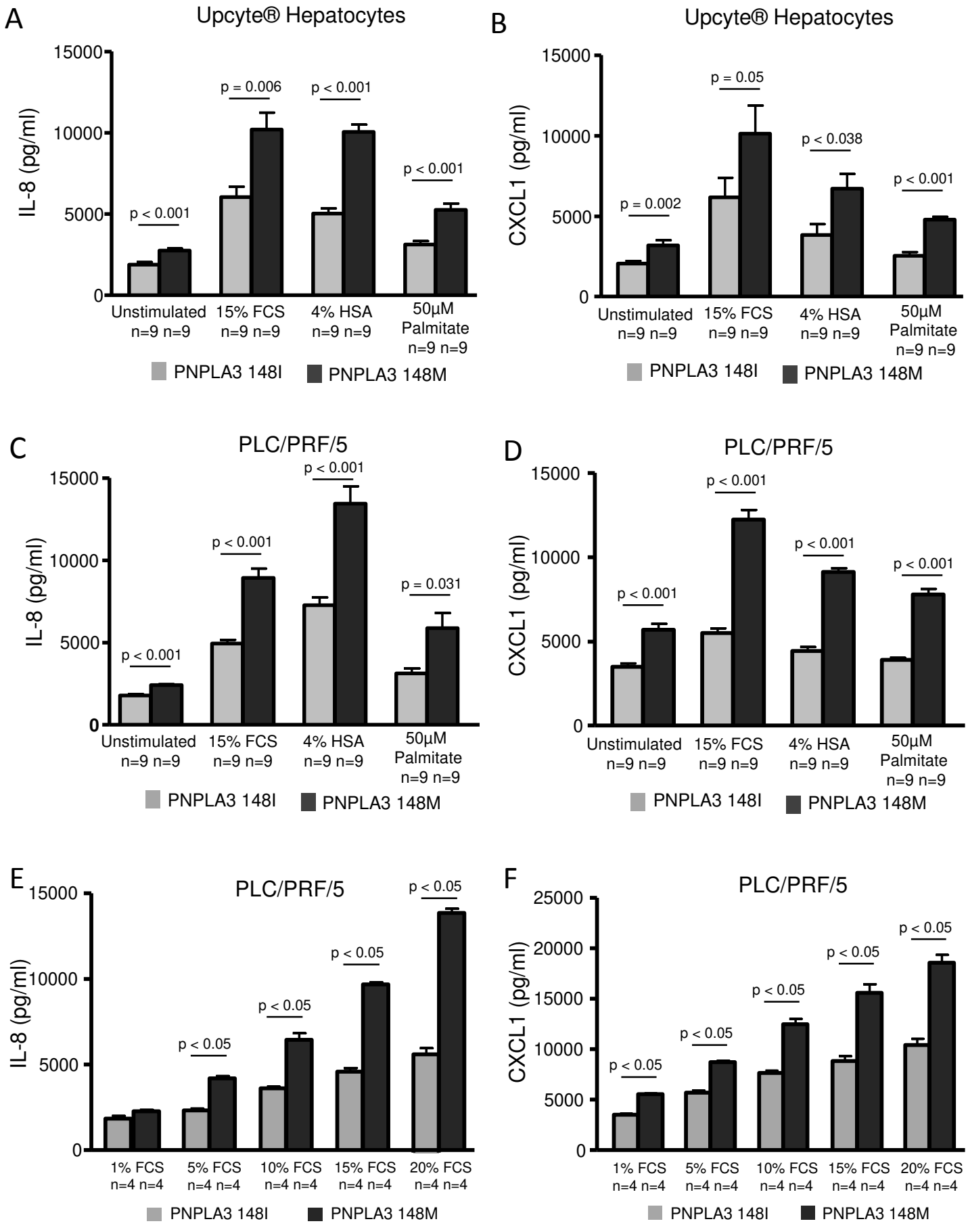

Figure 3

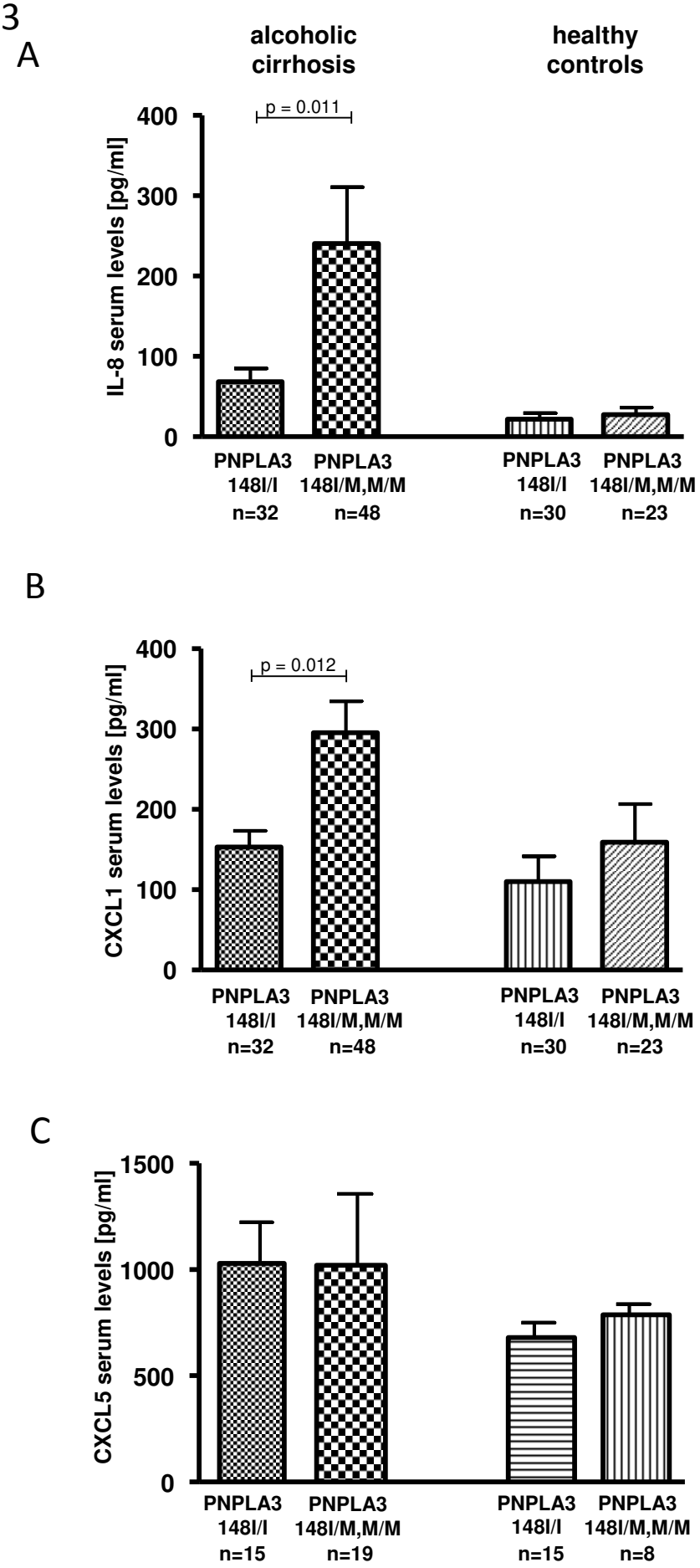
A
alcoholic cirrhosis
healthy controls
p = 0.011
IL-8 serum levels [pg/ml]
400
300
200
100
0
PNPLA3 148I/I n=32
PNPLA3 148I/M,M/M n=48
PNPLA3 148I/I n=30
PNPLA3 148I/M,M/M n=23
B
p = 0.012
CXCL1 serum levels [pg/ml]
400
300
200
100
0
PNPLA3 148I/I n=32
PNPLA3 148I/M,M/M n=48
PNPLA3 148I/I n=30
PNPLA3 148I/M,M/M n=23
C
CXCL5 serum levels [pg/ml]
1500
1000
500
0
PNPLA3 148I/I n=15
PNPLA3 148I/M,M/M n=19
PNPLA3 148I/I n=15
PNPLA3 148I/M,M/M n=8

Figure 4

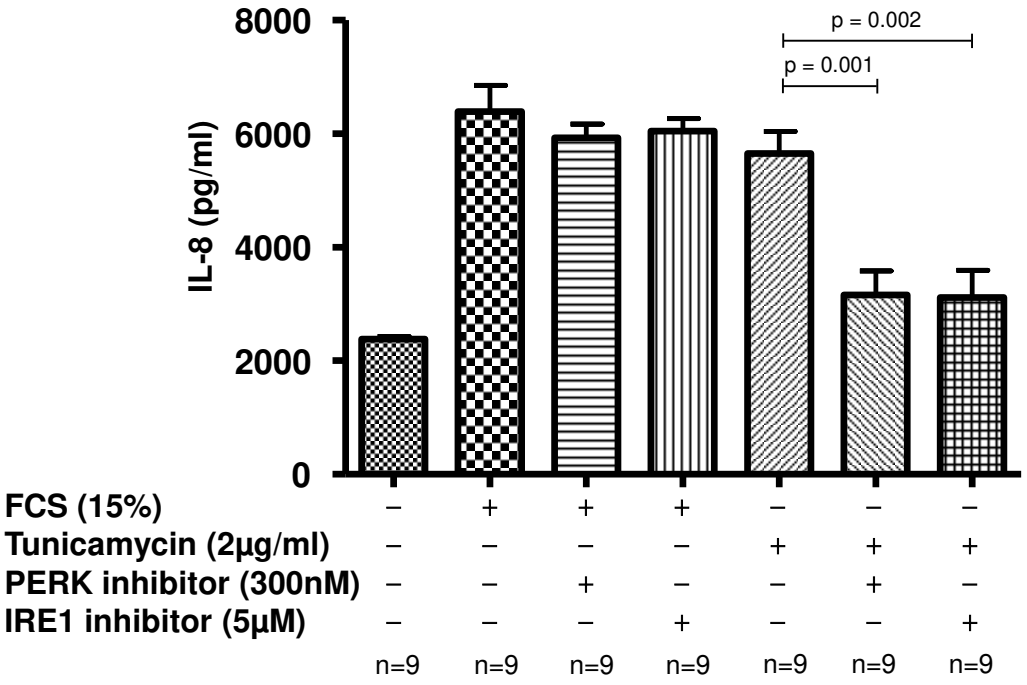
8000
6000
4000
2000
0
IL-8 (pg/ml)
p = 0.002
p = 0.001
FCS (15%) − + + + − − −
Tunicamycin (2µg/ml) − − − − + + +
PERK inhibitor (300nM) − − + − − + −
IRE1 inhibitor (5µM) − − − + − − +
n=9 n=9 n=9 n=9 n=9 n=9 n=9

Figure 5

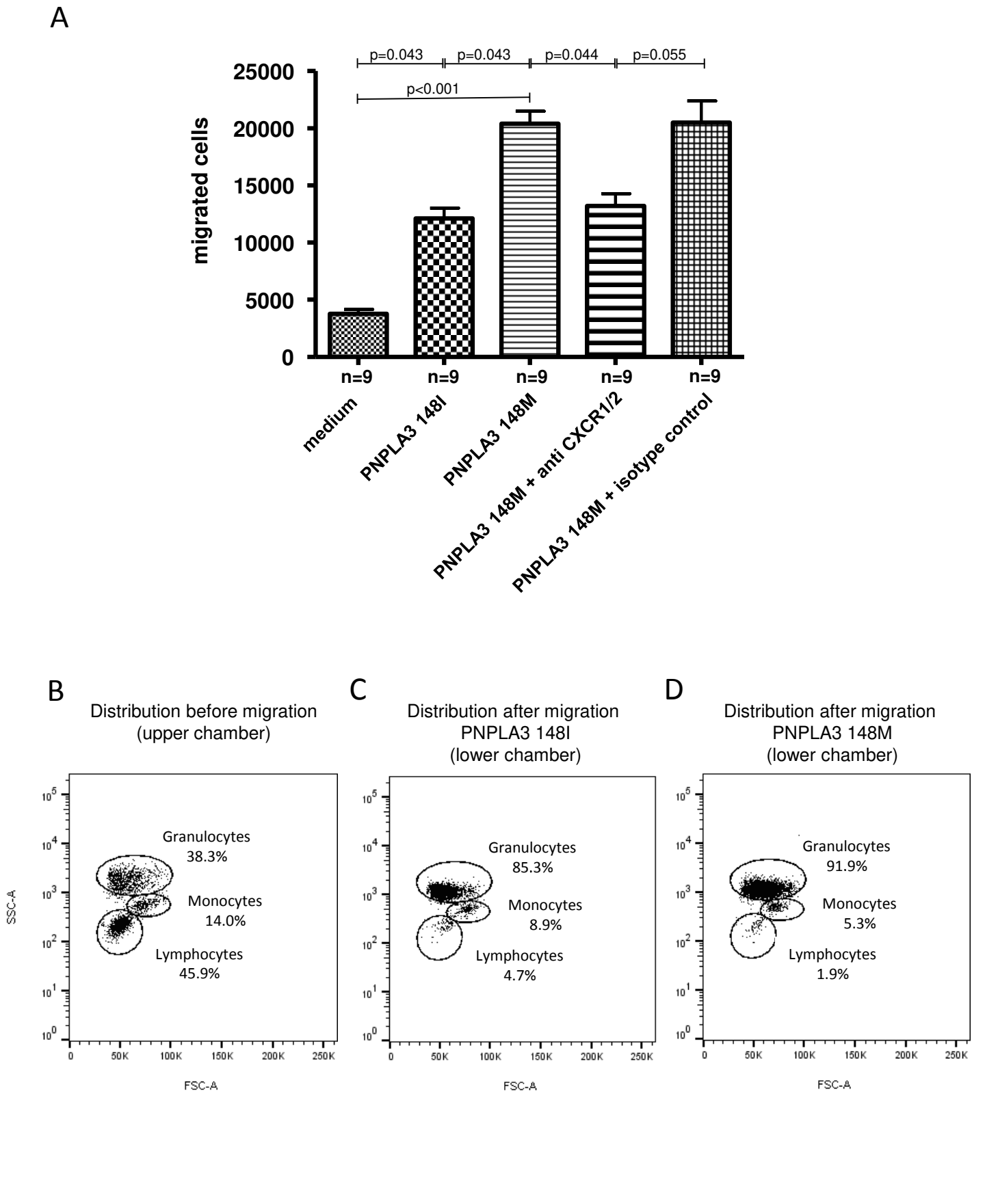
A
p=0.043
p=0.043
p=0.044
p=0.055
p<0.001
migrated cells
25000
20000
15000
10000
5000
0
n=9
n=9
n=9
n=9
n=9
medium
PNPLA3 148I
PNPLA3 148M
PNPLA3 148M + anti CXCR1/2
PNPLA3 148M + isotype control
B
Distribution before migration
(upper chamber)
Granulocytes
38.3%
Monocytes
14.0%
Lymphocytes
45.9%
C
Distribution after migration
PNPLA3 148I
(lower chamber)
Granulocytes
85.3%
Monocytes
8.9%
Lymphocytes
4.7%
D
Distribution after migration
PNPLA3 148M
(lower chamber)
Granulocytes
91.9%
Monocytes
5.3%
Lymphocytes
1.9%
SSC-A
FSC-A
0
50K
100K
150K
200K
250K

Figure 6

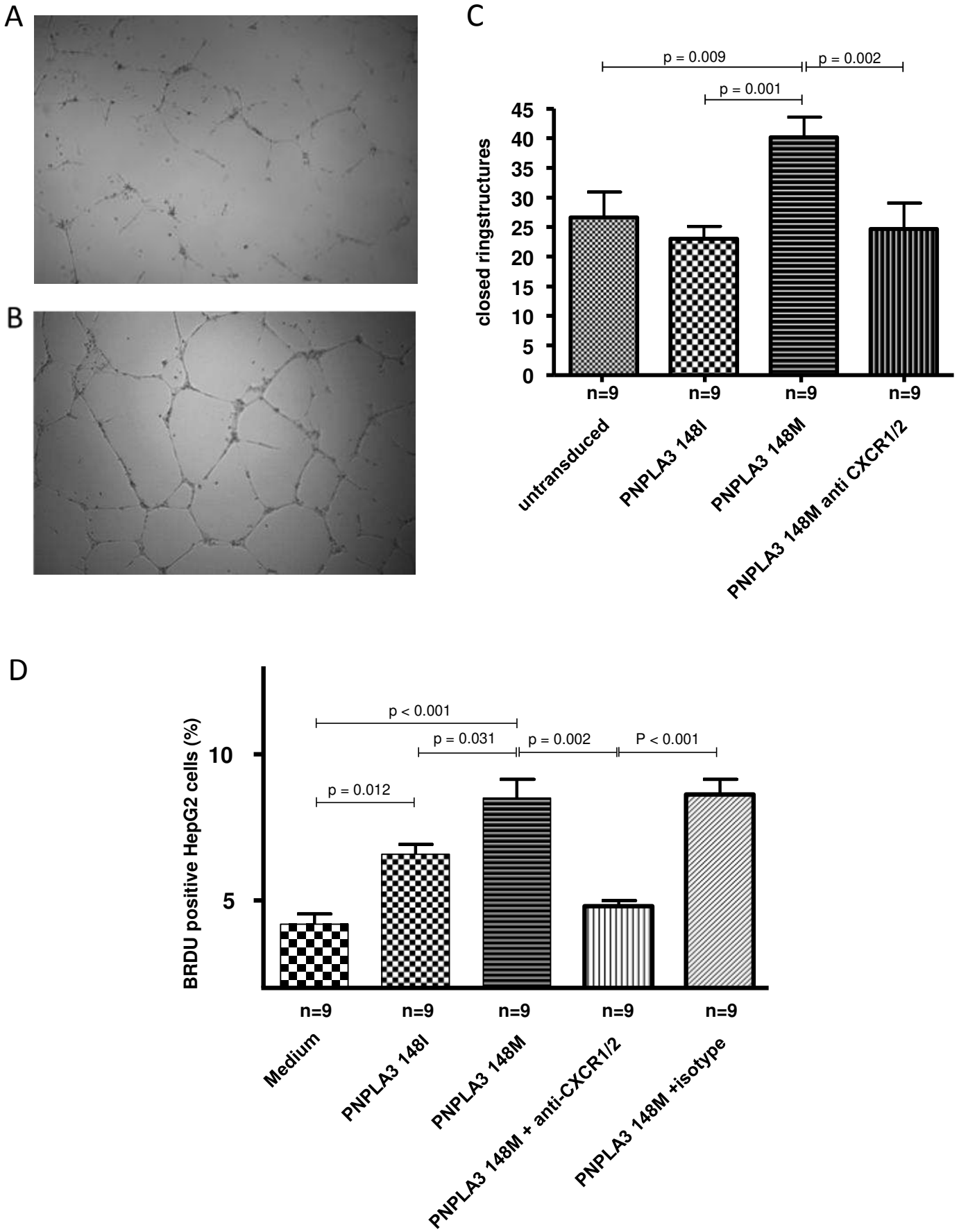
A
B
C
p = 0.009
p = 0.002
p = 0.001
closed ringstructures
45
40
35
30
25
20
15
10
5
0
n=9
n=9
n=9
n=9
untransduced
PNPLA3 148I
PNPLA3 148M
PNPLA3 148M anti CXCR1/2
D
p < 0.001
p = 0.031
p = 0.002
P < 0.001
p = 0.012
BRDU positive HepG2 cells (%)
10
5
n=9
n=9
n=9
n=9
n=9
Medium
PNPLA3 148I
PNPLA3 148M
PNPLA3 148M + anti-CXCR1/2
PNPLA3 148M +isotype

Table 1. Demographic and Clinical Data of the Study Groups

	Alcoholic cirrhosis (n=80)	Alcoholic cirrhosis PNPLA3 148I/I (n=32)	Alcoholic cirrhosis PNPLA3 148I/M,M/M (n=48)	P value	Healthy controls (n=53)
Median age, years (range)	57 (27-80)	56,5 (27-80)	57 (36-73)	0.53	34 (22-70) *
Gender (% male/female)	66.3 / 33.7	65.6 / 34.4	66.7 / 33.3	0.92	42 / 58 *
MELD, (Mean ± SD)	13.31 ± 5.1	12.7 ± 5.3	13.7 ± 5.0	0.44	-
Child-Pugh class A/B/C (%)	21/56/23	25/59/16	19/54/27	0.46	-
Bilirubin [mg/dl], (Mean ± SD)	3.04 ± 4.93	3.25 ± 4.81	2.90 ± 5.06	0.76	-
Platelet count [*10^3/µl], (Mean ± SD)	132 ± 64	137 ± 61	128 ± 66	0.54	-
ALT [IU/l], (Mean ± SD)	47.9 ± 46.6	54.4 ± 62.8	43.6 ± 31.7	0.31	-
AST [IU/l], (Mean ± SD)	75.5 ± 71.4	83.0 ± 98.7	70.5 ± 45.6	0.45	-

* $p<0.05$ vs. all other groups